Chapter 3
Brief Notes About Biofilms

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> ….There are several steps that we must take to optimize our lives in a city. The first is to choose the city in which we will live, then we must select the neighborhood in the city that best suits our needs, and finally we must make our home amongst the homes of many others. Occasionally, when life in the city sours, we leave….
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> P. Watnick, R. Kolter (2000) Biofilm, City of Microbes

Abstract The biofilm is a microbial community characterised by sessile bacterial cells strongly adherent to a substrate and/or an interface and incorporated in a polymeric matrix of microbial origin. In this condition, microbial cells exhibit an altered phenotype in comparison to corresponding free or planktonic forms. Life in a biofilm represents probably the prevailing mode of growth for microbes in several environments. After the development of new observation methods and the modification of different procedures, biofilms may be identified on known substrates and on new sites. The concept of biofilm has acquired the importance that still plays in the health sector, especially in areas where the use of invasive instruments and the continuous temporal localisation is expected. An important role is also played in the food industry because of the direct presence on food surfaces.

Keywords Alginate · Biofilm · Cellulose · Exopolysaccharide · Extracellular polymeric substance · *N*-acetylglucosamine · Succinoglycan

Abbreviations

CLSM   Confocal laser scanning microscopy
DNA   Deoxyribonucleic acid
ES   Exopolysaccharide
EPS   Extracellular polymeric substance

3.1 Biofilm: An Introduction

The biofilm is a microbial community characterised by sessile bacterial cells strongly adherent to a substrate and/or an interface and incorporated in a polymeric matrix of microbial origin. In this condition, microbial cells exhibit an altered phenotype with respect to growth rates and the gene transcription in comparison to the corresponding free or planktonic forms.

It has been reported that the bacterial adhesion triggers gene control mechanisms for the production of required molecules in the formation of biofilms (Donlan and Costerton 2002). The definition of biofilm involves not only a description of the morphostructural type but also biomolecular factors (Donlan 2002).

After the development of new observation methods such as scanning electron microscopy and the modification of different procedures, biofilms may be identified on known substrates and on new sites. Consequently, persistent and antibiotic-resistant infections have been explained in this way. In recent years, the 'biofilm phenomenon' has been analysed in different ways and situations. Many microorganisms have been the object of study, including:

- *Candida albicans* (Hawser and Douglas 1994; Sherry et al. 2014)
- *Escherichia coli* (Pratt and Kolter 1998)
- *Klebsiella pneumoniae* (di Martino et al. 2003)
- *Legionella pneumophila* (Atlas 1999; Declerck 2010; Declerck et al. 2007; Hindré et al. 2008; Murga et al. 2001; Storey et al. 2004)
- *Proteus mirabilis* (Holling et al. 2014; Jones et al. 2007; Moryl et al. 2014)
- *Pseudomonas aeruginosa* (Klausen et al. 2003; O'Toole and Kolter 1998; Ramsey and Whiteley 2004; Savoia 2014)
- *Staphylococcus aureus* (Kiedrowski et al. 2014; Islam et al. 2014; Wojtyczka et al. 2014).

3.1.1 Extracellular Polymeric Substances

As mentioned above, biofilm is a complex structure which is made of aggregates of microbial cells within a matrix of extracellular polymeric substances (EPS). EPS are high-molecular-weight compounds secreted by microorganisms into their environment.

The matrix structure constitutes the elastic part of the biofilm. Interstitial voids and channels separating micro-colonies contain a liquid phase, mainly constituted by water. The liquid phase is the viscous part of biofilms. The EPS matrix provides the biofilm with mechanical stability through these viscoelastic properties (Shaw et al. 2004) and the consequent functional and structural integrity. Physiochemical properties of biofilms are mainly determined by the qualitative and quantitative composition of EPS matrices (Flemming and Wingender 2010)

All major classes of macromolecules—polysaccharides, proteins, nucleic acids, peptidoglycan and lipids—can be present in a biofilm. Although extracellular polysaccharides are considered as the major structural components of the biofilm matrix, extracellular deoxyribonucleic acid (DNA) plays an important role in the establishment of biofilm structures (Whitchurch et al. 2002)

EPS are the construction material of bacterial settlements and either remain attached to outer cell surfaces or are secreted into its growth medium. These compounds are important in biofilm formation and cells' attachment to surfaces. EPS constitutes 50–90 % of the total organic matter in biofilms (Donlan 2002; Donlan and Costerton 2002; Flemming et al. 2000).

The development of microbial biofilms is observed virtually on all submerged surfaces in natural and industrial environments. Biofilms are also observed at interfaces as pellicles, or in the bulk of aquatic environments as flocs or granules (Marti et al. 2011).

Microorganisms synthesise a wide spectrum of multifunctional polysaccharides including intracellular, structural and extracellular polysaccharides or exopolysaccharides (ES). ES are high-molecular-weight polymers, the composition includes sugar residues which are secreted by a microorganism into the surrounding environment.

ES generally consist of monosaccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate and phosphate groups. Owing to the wide diversity in composition, ES have found multifarious applications in medical, pharmaceutical and food industries.

ES of some strains of lactic acid bacteria, including *Lactococcus lactis* subsp. *cremoris*, contribute a gelatinous texture to fermented milk products such as *Viili* (a yoghurt-like mesophilic fermented milk); these polysaccharides are also digestible (Ljungh and Wadstrom 2009; Welman 2009). An example for the industrial use of ES is the application of dextran in the Italian *panettone* and other breads in the bakery industry (Ullrich 2009).

Lembre and coworkers have reported various examples of ES and carbohydrates in bacterial biofilm (Lembre et al. 2012). The composition of ES can vary according to producing bacterial species (Sects. 3.1.1.1–3.1.1.3).

3.1.1.1 Alginate and N-Acetylglucosamine

Alginate, a polysaccharide extracted by brown algae and different bacteria such as *Azotobacter vinelandii* (Gorin and Spencer 1966) and *P. aeruginosa* (Davies et al. 1993; Davies and Geesey 1995; Linker and Jones 1964), has been extensively studied (Fig. 3.1). In detail, alginate is an ES with a relatively high molecular mass (10^4–10^6 g/ml). This compound consists (Gacesa 1998) of two uronic acid residues: β-D-mannuronate, also named M, and its C-5 epimer, α-L-guluronate, also named G (Fig. 3.1).
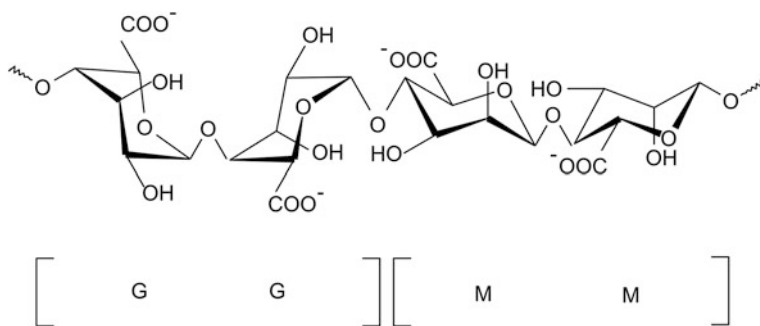
Fig. 3.1 Exopolysaccharides in biofilm matrices: structure of G- and M-alginates. G-structures mean 'poly-α-L-guluronate' while M-blocks are 'poly-β-D-mannuronate'. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

Another interesting ES, *N*-acetylglucosamine (Fig. 3.2), is produced by *E. coli*, *S. aureus* and *S. epidermidis* (Cerca and Jefferson 2008; Kaplan et al. 2004; Izano et al. 2008).

3.1.1.2 Succinoglycan

Succinoglycan is produced by *Alcaligenes faecalis* var. *myxogenes* 10C3, a microorganism our research group has isolated from soils. This microorganism produces a water-soluble and an insoluble extracellular polysaccharide. The former compound, succinoglycan, is composed of glucose, galactose, pyruvic acid and succinic acid (molar proportions are 7:1:1:1), with (β1-3)-, (β1-4)- and (β1-6)-glucosidic linkages. These polymers or oligomers are also produced by many *Agrobacterium* and *Rhizobium* strains (Harada 1983; Harada and Amemura 1979; Hisamatsu et al. 1982; Tomlinson et al. 2010).

3.1.1.3 Cellulose

Cellulose (Fig. 3.3) is the most abundant sugar polymer found on the surface of the planet Earth. It is found throughout the living world: in plants, animals, fungi and in bacteria such as *Salmonella* spp, *E. coli, Acetobacter, Agrobacterium* and *Rhizobium* (Matthysse et al. 2005; Solomon et al. 2005; Spiers and Rainey 2005).
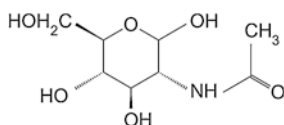

Fig. 3.2 Structure of *N*-acetylglucosamine. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure
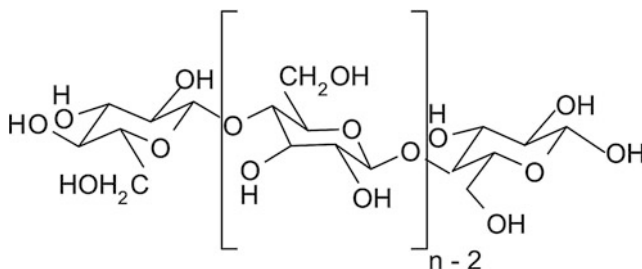
Fig. 3.3 Structure of cellulose. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

3.2 The Biofilm and Its Creation

As mentioned earlier, biofilms are an aggregation of microorganisms attached to and growing on a surface (Costerton and Stewart 2001). The formation and the subsequent development of biofilms are affected by many factors, including the specific bacteria strain (Borucki et al. 2003; Chae and Schraft 2000), material surface properties and environmental parameters such as pH, available nutrient levels and temperature (Donlan 2002).

The biofilm formation is a dynamical process consisting of five steps: (1) initial attachment, (2) irreversible attachment, (3) early development of biofilm architecture, (4) maturation and (5) final dispersion (Fig. 3.4).

3.2.1 Initial Attachment

The initial attachment of microorganisms can be active or passive, depending on their motility, the gravitational transportation of their planktonic species (free floating) or the diffusion of the surrounding fluid phase (Kumar and Anand 1998). Physical properties of the environment are essential for microorganism's attachment to the substratum, biofilm formation and microbial processes. The cell adhesion during this process strongly depends on the physiochemical properties of the
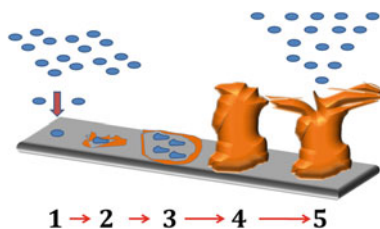

Fig. 3.4 Steps of biofilm development: (**a**) reversible attachment; (**b**) irreversible attachment; (**c**) micro-colony formation; (**d**) maturation; (**e**) final dispersion

bacterial cell surface (Begoude et al. 2007). At first, adherent cells possess only a small quantity of EPS; many of these life forms are capable of independent movement (O'Toole and Kolter 1998) by pilus-mediated twitching or gliding motility. The adhesion is reversible in this stage (Fig. 3.4).

3.2.2 Irreversible Attachment

The switch of the biofilm from the reversible to the irreversible state is related to the interaction between the presence of bacteria, EPS production and the surface (Fig. 3.4). In this step, cells lose their flagella-driven motility (Stoodley et al. 2002). After this irreversible attachment, a very strong force is required to remove the biofilm. For this reason, there is the need of chemicals such as enzymes, detergents or surfactants. McCoy and coworkers have shown that microbial adhesion strongly depends on hydrophobic–hydrophilic properties of interacting surfaces (McCoy and Brown 1998).

3.2.3 Micro-Colony Formation

Micro-colony formation results from the simultaneous aggregation and the increase of microorganisms (Fig. 3.4). As a result, the formation of biofilms is associated with the production of EPS, which helps to reinforce links between bacteria and the substratum. In addition, the colony is protected from any environmental stress (Donlan 2002).

Micro-colonies are discrete matrix-enclosed communities that may include cells of one or many species. Depending on the involved species, the micro-colony may be composed of 10–29 % cells and 75–90 % EPS- matrix (Costerton et al. 1987). Bacterial cells within the matrix are characterised by their lack of Brownian motion; the structural analysis of many micro-colonies often reveals a mushroom-like shape (Costerton et al. 1995).

Studies of bacterial species in natural systems have shown (McLean et al. 1997; Swift et al. 2001) that planktonic cells from the neighbouring medium can be included in this stage into the biofilm using the cell–cell communication (also named *quorum sensing*).

3.2.4 Maturation

After attachment to a surface, bacteria undergo further adaption to life in a biofilm. Two significant properties are often associated with this step: the increased EPS synthesis and the development of antibiotic resistance. In this step, bacteria develop other properties such as ultraviolet light—resistance, increased rates of genetic

exchange, altered biodegradative capabilities and the increased production of secondary metabolites (O'Toole et al. 2000). For example, the presence of protein from milk or meat can alter physicochemical properties of surfaces (charge, free energy, hydrophobicity, etc.), which further results in a greater bacterial attachment (Kumar and Anand 1998).

The biofilm maturation (Fig. 3.4) is the step where the living matrix develops into an organised structure which can be flat or mushroom-shaped (Fig. 3.4), depending on available nutrient sources (Klausen et al. 2003).

In order to reach structural maturity, periods of 10 days or more are required (Stoodley et al. 2002). Bacteria grow under sessile form in motley complex-enclosed micro-colonies scattered with open water channels (Davey and O' Toole 2000). Mature biofilms have also been evaluated and compared with chemostat cultures of *P. aeruginosa* by the DNA microarray technology. Results have shown that over 70 genes were altered, including genes encoding proteins involved in translation, metabolism, membrane transport and/or secretion, and gene regulation.

3.2.5 Dispersion

After the fourth stage, several attacked bacteria can leave the 'aged' biofilm individually or in groups and disperse in the environment. Results are the survival of these life forms and the colonisation of new niches. This detachment is a discontinuous and occasional process; it depends on various factors such as:

(a) Flow variations of the surrounding liquid
(b) The presence of chemicals or the modification of surface properties of cells or the colonised substrate
(c) Internal biofilm processes, such as endogenous enzymatic degradation
(d) The release of EPS or surface-binding protein.

These causes can occur at the same time; in addition, starvation is considered as a reason for the detachment and forces bacteria to search for a nutrient-rich environment (Srey et al. 2013). Every microorganism that breaks off can be transported in the environment and start again the process of biofilm formation (Fig. 3.4).

3.3 Some Environmental Factors Affecting Biofilm Formation

3.3.1 pH and Temperature

Bacteria respond to internal and external pH and temperature modifications with the variation of the activity and synthesis of proteins associated with many different cellular processes (Olsen 1993). A gradual increase in acidity determines good

chances of cell survival if compared to a sudden increase by the rapid addition of hydrochloric acid (Li 2001). This result suggests that bacteria contain mechanisms in place which allow the microbial population to adapt to small pH changes (Garrett et al. 2008). Bacteria have membrane-bound proton pumps which extrude protons from the cytoplasm to generate a trans-membrane electrochemical gradient, i.e. the proton motor force. The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH (Booth 1985). Large variations of external pH values can overwhelm such mechanisms and have a biocidal effect on microorganisms. The optimum pH for polysaccharide production depends on the individual species; however, it has been reported that approximate pH values of around 7 are good for most bacteria (Oliveira et al. 1994).

3.3.2 Surface Topography

The roughness of substrates is known to be one of the key factors in determining the extent of bacterial colonisation (Crawford et al. 2012; Oh et al. 2009). Also, it plays a significant role in the attachment process, particularly when superficial irregularities are comparable to microbial sizes and can provide shelter from unfavourable environmental factors (Mitik-Dineva et al. 2008). Moreover, levels of bacterial adhesion are determined by the surface topography. The relationship between surface roughness and the attachment (and growth) of bacteria may vary depending on involved microbial species. Furthermore, once bacteria have colonised a surface, the resistance of biofilms to the removal by simple friction increases in proportion to the superficial roughness (Preedy et al. 2014).

3.3.3 Hydrophobicity and Hydrophilicity

Hydrophobicity plays an important role in the microbial attachment on surfaces: this factor has an impact on the temporal length of cells association with the substratum. The hydrophobicity of cell surfaces is also important in adhesion because the hydrophobic interaction tends to augment when non-polar properties of each surface increase. (Alsteens et al. 2007; Faille et al. 2002; van Oss 1995, 1997)

3.4 Food and Food Industry

The role of biofilms in the food sector is relevant. Many authors have demonstrated the presence of microorganisms organised in biofilms directly on the food as well as on different materials that will be in contact with it. Germs can adhere strongly to glass or stainless steel tools and ceramic and polypropylene surfaces, with the

resulting difficult eradication (Adetunji et al. 2014; Balsanelli et al. 2014; Regina et al. 2014).

In environments of food processing bacteria with other organic and inorganic molecules, such as milk—or meat—proteins, a conditioning film can be formed. This film, composed of many complexes, can be displaced by diffusion or transported by liquid flows. The accumulation of molecules at the solid–liquid interface on the contact surface of the food can lead to a high concentration of nutrients with respect to the fluid phase. The increased level of nutrients deposited on contact surfaces can favour the formation of biofilm, acting as a conditioning film.

The adhesion of bacteria to food or food- contact surfaces represents a serious hygiene problem; it can determine economic losses due to possible food spoilage. In addition, some studies have demonstrated the persistence of some foodborne pathogens on surfaces in contact with foods and some germs has been recently added to the existing list: *Salmonella* (de Oliveira et al. 2014; Lianou and Koutsoumanis 2012, 2013; Patel et al. 2013; Schonewille et al. 2012; Wang et al. 2013); *Listeria monocytogenes* (Barbosa et al. 2013; Borucki et al. 2003; Ferreira et al. 2014; Valderrama and Cutter 2013); *Yersinia enterocolitica* (Zhou and Yang 2011; *Campylobacter jejuni* (Joshua et al. 2006; Reeser et al. 2007; Teh et al. 2010, 2014); *Aeromonas hydrophila* (Elhariry 2011; Jahid et al. 2013); *E. coli* and *E. coli 0157: H7* (Dourou et al. 2011; Gomes et al. 2014; Silagyi et al. 2009; Simpson Beauchamp et al. 2012); *S. aureus* (Gutiérrez et al. 2012). The persistence and the accumulation of germs in biofilms could be a source of subsequent contamination, leading to a reduced shelf life of the product. The transmission of pathogens may also occur by produced aerosols during surface-cleaning processes in food plants (Italian Institute of Packaging 2009; Parisi 2011, 2012; Steinka 2015).

One of the main objectives of the food industry is the production of safe and healthy products with good organoleptic quality. Consequently, the growth of microorganisms has to be monitored in order to minimise the risk of food contamination by germs into food plants (environmental contamination).

Moreover, monitoring activities have to consider methods of production and handling of food products, involving frequent contact of raw materials, semi-finished and finished products with work surfaces and utensils. It can be assumed that the transfer of microbial flora with pathogenic or degrading features to foods may be easily observed when considering work surfaces, utensils, packaging materials and correlated machinery equipment (Parisi 2013).

The presence of nutrient residuals on surfaces can promote the formation of biofilms; on the other hand, the simple presence of organic substance in drinking water may be sufficient, when surfaces are washed.

From the industrial viewpoint, biofilms represent a problem that mainly concerns food industries: breweries, dairy companies, meat industries, etc. (Chen et al. 2007; Frank et al. 2003; Jessen and Lammert 2003; Simões and Vieira 2009; Simões et al. 2010; Somers and Wong 2004).

With reference to food production environments, bacteria and chemical compounds (organic and inorganic molecules such as milk or meat proteins) can adhere to non-food surfaces with the consequent formation of conditioning films. Organic

and inorganic molecules are transported together with microorganisms on surfaces by diffusion or by the turbulent flow of liquids in some cases (Poulsen 1999; Sharma and Anand 2002). In these systems, the amount of residual nutrients with strong adherence to surfaces is notable and can condition the surface itself (Hood and Zottola 1997). Basically, the increase of nutrients promotes biofilm formation (Jeong and Frank 1994).

The creation of biofilm on food-contact materials leads to a number of hygienic and economic problems at the same time: in fact, biologic contamination can cause food deterioration before of the labelled expiration date (Parisi 2002a, b, c).

On the other hand, it is known that surfaces and tooling in contact with foods must be made with easily cleanable and disinfectable materials (Gurnari 2015). The use of steel and hardness plastics has been promoted for this reason, excluding porous materials such as wood.

For example, dirty or unclean equipment are considered as the main sources of contamination (with airborne microflora) of milk and milk products. The accumulation and the persistence of biofilms can cause post-process contamination, shortening shelf-life values of food products (Jin and Zhang 2008; Zottola and Sasahara 1994).

The contamination may occur between packaging and consumption steps at any time. According to recent literature on polyethylene terephthalate (common plastic bottles for drinks are made with this plastic matter), bacteria can adhere tenaciously and constitute biofilms in less than 24 h; on the other hand, polypropylene (containers and plates) is not easily contaminated by moulds and bacteria (Byun et al. 2007).

The modern equipment used in food production processes are made of different materials such as stainless steel, glass, plastic, rubber and polytetrafluoroethylene (Teflon®); the degree of hydrophobicity of these surfaces can influence microbial adhesion (Sinde and Carballo 2000; Teixeira et al. 2005).

Food contamination may appear at different stages of the production chain; in particular, certain perishable products such as milk have to be considered. Milk can be contaminated during the milking stage by faccal contamination or the simple use of contaminated water. With specific reference to milk and its derivatives, saprophytic or pathogens bacteria may creep under the seals of transformation or packaging machinery and contaminate food products and/or the final container (or single parts), thus leading to the formation of biofilm. Should the contamination concern packaging materials, this phenomenon and the consequent biofilm development would occur after pasteurisation. Surfaces such as shelves and walls can be indirectly source of contamination: germs can be transported from the air, as well as by operators' hands.

The design of equipment and the careful selection of materials of work surfaces are of crucial importance in preventing the formation of biofilms (Gurnari 2015). The most suitable material is stainless steel (it can be easily cleaned using common detergents).

The prerequisite for an efficient sanitation programme is correlated with the so-called hygienic design (Gurnari 2015). Corners, cracks, joints, gaskets and

crevices are vulnerable points for the accumulation of biofilm. Should the design of equipment and work surfaces be improper, any sanitation programme would represent possible contamination problems. Therefore, best strategies for the control of food-contact surfaces can be expressed as follows:

(a) The design of equipment should aim towards an efficient hygiene program, perform a proper cleansing and implement a good sanitation
(b) An effective cleaning programme removes undesired materials from food-contact surfaces, including microorganisms, foreign matter and any residues of detergent.

3.5 Strategies of Biofilm Control

3.5.1 Physical Methods

With exclusive reference to the removal of biofilms in food industries, the most innovative methods of physical nature include:

(a) High-frequency ultrasound
(b) High-frequency pulsed electric fields (the concomitant use of organic acids can be considered)
(c) Low-frequency electric fields (with the aim of increasing the effect of biocides).

Recently, the use of electric fields associated with antibiotics has been defined effective when speaking of *Pseudomonas* biofilm control.

3.5.2 Chemical Methods

The effective elimination of microorganisms on surfaces is important before disinfection procedures. Germs become much more sensitive to disinfectants when the biofilm is removed from surfaces. Even the mechanical or chemical break of polysaccharide matrices is important for the control of biofilms. Among the detergents with effective power against biofilms, chelating substances should be mentioned: these compounds include ethylenediaminetetraacetic and aminoethylene glycolethyl tetracetic acids. Some oxidising disinfectants are able to depolymerise ES, including acid peracetic acid, chlorine, iodine and hydrogen peroxide. These substances allow the detachment of biofilms formed by certain types of bacteria from surfaces.

3.5.3 Biological Methods

An innovative strategy for the control of biofilms consists in the adsorption of bioactive molecules such as bacteriocins and enzymes on food-contact surfaces. For example, nisin has been adjudged effective when speaking of adherence reduction of *L. monocytogenes* to surfaces (Saà Ibusquiza et al. 2011). The use of peculiar lactic bacteria cultures and their extracts allows the inhibition of degrading microorganisms and pathogens on food-contact (poultry products). As regards enzymes, they have been defined very efficient as detergents because of the inactivation of biofilm exocellular polymers and the enhancement of biofilm removal.

3.6 Biofilm in Hospital Setting and Antibiotic Resistance

It has to be noted that the chronicity of persistent bacterial infections is due to bacterial biofilm formation, in contrast with planktonic bacteria found in acute infections (Bjarnsholt 2013).

The epidemiologic evidence has clearly shown that biofilms have a role in infectious diseases (cystic fibrosis, periodontitis, bloodstream and urinary tract infections) as a result of indwelling medical devices. The process may be particularly relevant for immunocompromised patients who lack the ability to contrast invading organisms. Beyond the evidence, however, the exact processes by which biofilm-associated organisms elicit disease in the human host are poorly understood. Suggested mechanisms include the following steps:

(1) Detachment of cells or cell aggregates from indwelling medical device biofilms, resulting in bloodstream or urinary tract infections
(2) Production of endotoxins
(3) Resistance to the host immune system
(4) Provision of a niche for the generation of resistant organisms (through resistance plasmid exchange).

One of the most fascinating defence mechanisms of a biofilm is based on a peculiar type of intercellular signalling, the so-called *quorum sensing*. Some bacteria release a molecular signal, also called inductor. These molecules are acylated homoserine lactone for Gram-negative bacteria and peptic substances (peptides, amines, amino acids) for Gram-positive microorganisms.

As the cell density rises, the concentration of these molecules increases. Inductors interact with specific receptors in each cell to activate *quorum sensing* genes and start a cascade of events, thus causing the expression or repression of numerous other genes on the bacterial chromosome.

The structure and physiological attributes of biofilm-producing organisms confirm the inherent resistance to antimicrobial agents such as antibiotics, disinfectants

and germicides. The mechanisms responsible for resistance may be described approximately as follows:

1. Loss of penetration power of antibiotics through the biofilm matrix
2. Percentage of altered growth of germs which constitute the biofilm
3. Other physiological changes due to the mode of growth of the biofilm.

Antibiotic molecules must diffuse into the biofilm matrix to inactivate included cells. Extracellular polymeric substances that constitute the matrix represent a barrier to the diffusion of these molecules so as to hold both the percentage of the transport of molecules to the inside of biofilm and the interaction of the antimicrobial matter with the material from the matrix. A loss of power of penetration of ciprofloxacin against *P. aeruginosa* has been demonstrated (Suci et al. 1994); in other words, the normal sterilisation of surfaces (needed time: 40 s) would require 21 min when surfaces are covered by biofilms. Bacterial cells have been reported to be 15 times more sensitive to tobramycin if compared to cells of an intact biofilm (Hoyle et al. 1992).

Another mechanism has been proposed to explain the resistance to antimicrobial agents. In brief, cells associated with the biofilm may grow significantly more slowly than planktonic cells with the delayed absorption of antimicrobial. Anwar and coworkers have demonstrated that old cells (life: ten days) of *P. aeruginosa* biofilms were much more resistant to tobramycin and piperacillin biofilm cells (Anwar et al. 1989) if compared to younger cells (life: two days).
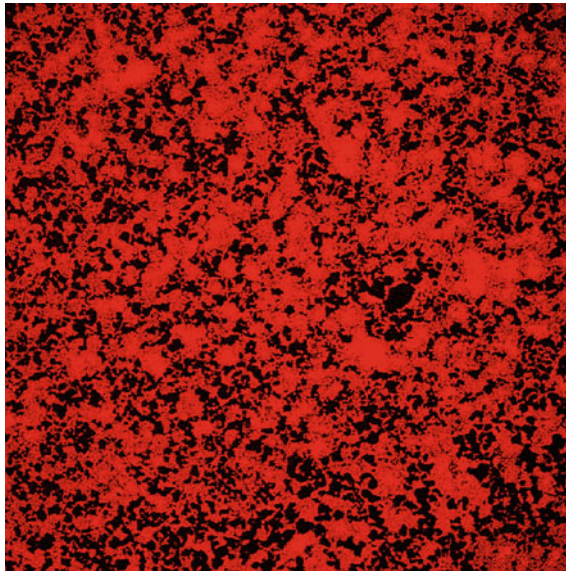
Fig. 3.5 A biofilm formed by *S. aureus*, grown on a glass surface (CLSM image; 2014, Laganà P, Delia S., University of Messina, Italy)

A dosage of 500 mg of piperacillin + tobramycin (5 mg/ml) can inactivate completely planktonic cells and biofilms of young cells. On the other side, older cells have been reduced only by approximately 20 % in the same condition.

3.6.1 Measurement and Observation of Biofilms

Biofilm growth and structure has been measured using diversified methods: fluorescence, transmission electron, scanning electron, atomic force and confocal laser scanning microscopy (CLSM). In relation to the CLSM system, our research group has used a TCS SP2- laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), equipped with argon-krypton laser and coupled to a Leica DMIRB-inverted fluorescence microscope. The use of CLSM requires the use of fluorescent stains as propidium iodide, a nucleic acid intercalating and fluorescent agent (Sigma). A biofilm produced by *S. aureus*, grown on glass and analysed by CLSM, is shown in Fig. 3.5.

Numerous methods are used with the aim of quantifying biofilm production (Sternberg et al. 2014). Microtiter plates are among the most frequently used biofilm model systems (Djordjevic et al. 2002; Elkhatib et al. 2014; Kwasny and Opperman 2010; Pierce et al. 2010). In these systems, biofilms are either grown on the bottom and the walls of the microtiter plate (most commonly a 96-well plate); results are usually read using a spectrometer (Fig. 3.6) (Table 3.1).
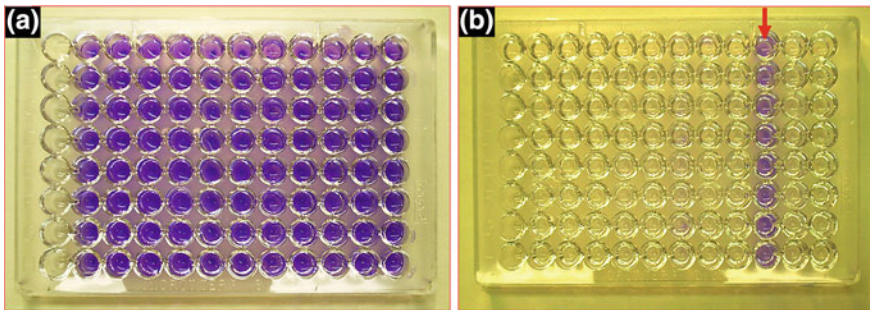
Fig. 3.6 (**a**) A microtiter plate where *S. aureus* and *P. aeruginosa* are incubated after 45 min at room temperature with 1 %-crystal violet. (**b**) A microtiter plate after washing and addition of 95 % ethanol. The arrow shows a strip with a strain of *S. aureus* (Laganà P and Delia S, University of Messina, Italy)

Table 3.1 Summary table of the main strategies for the management and control of biofilms

Classification of operative procedures for biofilm control	Definition of strategies
Physical Methods	High intensity-magnetic fields
	High energy-pulsed electric fields
	Low energy-electric fields
Mechanical	Brushing
Methods	Scraping
Chemical	Chelating agents
Methods	Peracetic acid
	Chlorine
	Iodine
	Hydrogen peroxide monolaurate
	Cetylpyridinium chloride
	Other chemical agents
Biological	Bacteriocins (e.g.,nisin)
Methods	Lactobacillaceae
	Microbial enzymes: protease, alpha-amylase, beta-gluconase, endo-glycosidases (endo-H), exo-polysaccharidase, etc.

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